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(54) **Methods of regeneration of medicago sativa and expressing foreign DNA in same**

Verfahren zur Regeneration von Medicago sativa und Expression fremder DNS darin

Procédé de régénération de Medicago sativa et expression d'ADN étrangères dans cette Medicago sativa

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(73) Proprietor: **PIONEER HI-BRED INTERNATIONAL, INC.**
Des Moines, Iowa 50309 (US)

(72) Inventors:
- **Buising, Charisse Marie**
  Iowa 50325 (US)
- **Tomes, Thomas**
  Iowa 50061 (US)
- **Schmidt, Janice**
  Saskatoon, Saskatchewan (CA)

(74) Representative: **Goldin, Douglas Michael et al**
J.A. KEMP & CO.
14 South Square
Gray's Inn
London WC1R 5LX (GB)

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Description

BACKGROUND OF THE INVENTION

[0001] Genetic transformation of plants has been one of the major advances achieved in biotechnology and its contributions to producing improved plants, improved crops, and consequently improved availability of food worldwide has been widely recognized. In certain plants, however, transformation has been especially difficult to achieve, and transformation of the valuable forage crop alfalfa, Medicago sativa has been inhibited by the peculiarities of the plant.

[0002] Transformation of alfalfa has been hampered primarily by two major limitations: constraints imposed by the method of transformation, and the poor regeneration from tissue and cell cultures of many alfalfa varieties.

[0003] The first limitation occurs because alfalfa is presently primarily transformed through the use of Agrobacterium tumifaciens. Agrobacterium exhibits host strain specificity and only certain Agrobacterium strains will infect a few alfalfa genotypes. The ability to transform alfalfa is considerably limited as a result. The second major inhibition of transformation of alfalfa is its own poor regeneration frequency. Only a few varieties exhibit even modest regeneration, and those elite varieties providing superior performance in the field are notoriously poor regenerators. The combination of these two problems has created a considerable bottleneck in achieving transformation of the plant.

[0004] Alfalfa exhibits other traits setting it apart from many crop plants. It is an autotetraploid and is frequently self incompatible in breeding. When selfed, the pollen may not germinate or, when it does, later stops germinating. Thus producing a true breeding parent for hybrids is not possible, which complicates breeding substantially.

[0005] It has been determined that there are nine major germplasm sources of alfalfa: M. falcata, Ladak, M. varia, Turkistan, Flemish, Chilean, Peruvian, Indian, and African. Culture of explant source tissue, such as mature cotyledons and hypocotyls, demonstrates the regeneration frequency of genotypes in most cultivars is only about 10 percent. Seitzkris, M.H. and E.T. Bingham, In vitro Cellular and Developmental Biology 24 (100:1047-1052 (1988). Efforts have been underway to improve regeneration, and have included attempts at asexual propagation to maintain individual genotypes which possess the regeneration trait. Further, propagation by asexual methods is not practical if many genotypes are involved. Bingham and others have attempted to avoid this problem by recurrent selection. In the first cycle, regenerating genotypes were selected, crossed and recycled until regeneration was improved to 60 percent or better. The result of this was the development of Regen-s, in which two-thirds of the plants were capable of regeneration from callus tissue. E.T. Bingham, et. al., Crop Science 15: 719-721 (1975).

[0006] Additionally, researchers believe that somatic embryogenesis in alfalfa is inheritable, and is controlled by relatively few genes. Efforts at improving regeneration have thus been directed towards isolation of the genetic control of embryogenesis, and breeding programs which would incorporate such information. See, e.g. M.M. Hernandez-Fernandez, and B.R. Christie, Genome 32:318-321 (1989); I.M. Ray and E.T. Bingham, Crop Science 29:1545-1548 (1989). This is complicated by the characteristics of alfalfa noted above. This invention relates to improvements in transformation and regeneration of alfalfa by departing from these previous approaches.

[0007] Successful transformation of the legume Glycine max (soybean) has previously been achieved by employing transformation-regeneration procedures involving microprojectile bombardment of immature embryos or embryonic axes isolated therefrom (Christou et al., Plant Physiol. (1989) 87, 671-674; McCabe et al., Bio/Technology (1988) 6, 923-926; Christou et al., Proc. Natl. Acad. Sci. USA (1989) 86, 7500-7504). It has now been found that limitations in regeneration of alfalfa are overcome by selecting immature cotyledons up to 25 days past pollination for initiation of somatic embryogenesis. By combining such regeneration with use of microprojectile bombardment to introduce foreign DNA into immature cotyledon cells of alfalfa or somatic embryos derived therefrom even elite varieties of alfalfa can be regenerated and transformed.

DESCRIPTION OF THE DRAWINGS

[0008] Fig. 1 is a map of plasmid pPHI251

[0009] Fig. 2 is a map of plasmid pPHI256.

[0010] Fig. 3 is a graph showing time course harvest results plotting age of the cotyledon on the x axis and regeneration response on the y axis.

[0011] Fig. 4 is a graph of regeneration using immature cotyledons (hashed bar) and mature cotyledons (solid bar) of varieties listed.

[0012] Fig. 5 is a map of plasmid pPHI413.

DESCRIPTION OF THE INVENTION

Microprojectile bombardment

[0013] Microprojectile bombardment in order to transform plant cells is known to those skilled in the art. The general process has been described by T.M. Klein, et al. Proc. Natl. Acad. Sci. USA 85:4305-4309 (1988). The basic process includes coating DNA onto small high density particles, microprojectiles, which are then placed into the particle gun or helium gun apparatus and accelerated to a high velocity in order to penetrate plant cell walls and membranes and carry the DNA or other substance into the interior of the bombarded cell.

[0014] Previous work involved delivery of foreign genes through this method into intact plant of tobacco tissue, but its application to the economically important species alfalfa has not been successfully accomplished. Tomes, et al. Plant Molecular Biology 14:261-268 (1990). Microprojectile bombardment of alfalfa to achieve transformation has not been previously reported.

[0015] Introduction of DNA into a plant is demonstrated at first by transient expression. Short term expression is noted by confirming the presence of the DNA within the plant cells 24 to 48 hours after bombardment. When expressed up to 72 hours after bombardment it is demonstrated that the DNA has been delivered via the particle gun or other method and that the DNA vector functions. When continuing to be expressed two to eight weeks after bombardment, it may be concluded the DNA is persistent and likely integrated into the plant genome. Its ability to persist at this point shows it has survived attack from nucleases which typically would attack unprotected foreign DNA. When the R_o plants are recovered, continuing expression is further indication that stable transformation into the plant cells has occurred. Southern analysis allows confirmation of this. When crossed and the R_1 generation analyzed, expression and inheritibility of the DNA is further confirmed.

[0016] A variety of plant cell sources can be used for transformation by microprojectile bombardment. Hypocotyls, cotyledons of mature seed and petioles are plant tissue which can be subjected to bombardment. The applicant has discovered that when immature cotyledons up to 25 days past pollination are used, satisfactory transformation results. While not wishing to be bound by any theory, it is proposed that immature cotyledons may be a better source of tissue for bombardment because the cells to be bombarded are those which are capable of giving rise to plants.

[0017] Tissue culture was also optimized for the maximum regeneration possibilities. In the experiments described below, Regen-S, was used. As noted supra, Regen-S is known for its improved regeneration potential. Set forth below are tissue cultures which were employed. The most important factor in tissue culture optimized for regeneration is high concentration of 2, 4-dichlorophenoxyacetic acid (2,4-D) as compared to a low concentration of kinetin. Tissue/organ culture is described generally by Atanassov and Brown in Plant Cell Tissue Organ Culture 4: 4:111-122 (1985).

CULTURE MEDIA

[0018] The following describes media used in regeneration of transformed and non-transformed alfalfa. It is to be understood that those skilled in the art could use media which varies considerably from these media and fall within the scope of the invention. The description is given by way of example.

Gamborg's Based Medium

[0019] Gamborg's B-5 medium is a widely used medium for culture of plant species. It is well known to those skilled in the art and is described in detail at 0.L. Gamborg, R.A. Miller, K. Ojima, Exp. Cell. Res. 50:151-158 (1968). It forms a component of media listed below.

Modified B5 Medium

[0020] This medium is described at Atanassov, A. and Brown, D.C.W. Plant Cell Tissue and Organ Culture 3:149-162 (1984). A typical mixture is that formulated by GIBCO Laboratories and include: 1 mg/l 2,4-D, 0.2 mg/l kinetin, 30 g/l sucrose, 3000 mg/l KNO_3, 895 mg/l $CaCl_2$, 800 mg/l 1-glutamine, 500 mg/l $MgSO_4 \cdot 7H_2O$, 100 mg/l serine, 10 mg/l L-glutathione, 1 mg/l adenine, with the modification that was used instead of gelrite reported in Atanassov, 9 g/l bacto agar. It forms a component of media listed below.

MS Medium

[0021] This medium is well known to those skilled in the art and is described in detail at T. Murashige and F. Skoog, Physiologia Plantarum 15:473-497 (1962). A typical mixture that formulated by Gibco Lab and includes:

Component	mg/l
NH_4NO_3	1650.0
KNO_3	1900.0
$CaCl_2 \cdot 2H_2O$[a]	440.0
$MgSO_4 \cdot 7H_2O$[b]	370.0
KH_2PO	170.0
Na_2 EDTA	37.3
$FeSO_4 \cdot 7H_2O$	27.8
H_3BO_3	6.2
$MnSO_4 \cdot H_2O$	16.9
$ZnSO_4 \cdot 7H_2O$	8.6
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025

Blaydes Medium and Modifications

[0022] This well known medium to those skilled in the art is described in detail at D.F. Blaydes, Physiol. Plant. 19: 748-753 (1966).

[0023] BO (basal Blaydes medium) contains per liter: 300 mg KH_2PO_4, 100 mg KNO_3, 1 g NH_4NO_3, 347 mg Ca $(NO_3)_2 \cdot 4\ H_2O$, 35 mg $MgSO_4 \cdot 7\ H_2O$, 65 mg KCl, 0.8 mg KI, 1.5 mg $ZnSO_4 \cdot 7\ H_2O$. 1.6 mg H_3BO_3, 4.4 mg $MnSO_4 \cdot H_2O$, 2 mg glycine, 0.1 mg thiamine hydrochloride, 30 g sucrose, 10 g (5.57 9 $FeSO_4$;$7H_2O$ in 500 ml hot distilled water with 7.45 g Na2EDTA in 500 ml hot distilled water with pH to 5.9-6.0.

[0024] BII medium is the same as BO, but contains 2 mg/l each NAA, Kinetin, and 2,4-D.

[0025] BOi2Y is the same as BO, but contains 100 mg/l inositiol and 2 g/l bacto yeast extract. After embryo induction, explants must be removed from exposure to 2,4-D. 2,4-D appears to inhibit embryo development.

Schenk and Hildebrandt (SH) medium

[0026] This medium is well known to those skilled in the art and is described in detail at B.V. Schenk and A.C. Hildebrant, Can. J. Bot. 50:199-204 (1975). SHII contains 9.05 μM 2,4-dichlorophenoxy acetic acid (2,4-D) and 9.30 μM kinetin

Modified SH medium

[0027] This medium is well known to those skilled in the art and is described in detail at D.H. Mitten, S.J. Sato, and T.A. Skokut, Crop Sci. 24:943-945 (1984). Modified SH medium contained: 25 μM α-naphthaleneacetic acid (NAA) and 10 μM kinetin, callus was transferred to SH medium containing 50 μM 2,4-D and 5 μM kinetin, transferred 3 days later to regeneration medium containing BOi2Y.

[0028] The following is presented merely as examples and are not intended to limit the scope of the invention.

[0029] In each of the experiments set forth below, Regen-S, as described above, was employed. This variety is known for its high regeneration potential. Genes encoding the Alfalfa Mosaic Virus coat protein (AMVcp), Phosphinotricin Acetyl Transferase (referred to here as BAR), Neomycin Phosphotransferase (NPTII) and β-glucuronidase (GUS), were transformed into this genotype using a DuPont PDS 1000~ particle gun. The alfalfa mosaic virus coat protein may protect plants from AMV pathogens, BAR inactivates the nonselective herbicide phosphinotricin, present in Basta® medium and NPTII inactivates kanamycin. Plasmid pPHI251 encoding for NPTII, and AMVcp was used. A map of this plasmid is shown in Figure 1. Plasmid pPHI256 was separately used as indicated below in coding for BAR, AMVcp, and GUS. A map of this plasmid is found at Figure 2.

EXPERIMENT 1

Alfalfa Mature Cotyledon Particle Gun Transformation on Basta® Selection

[0030]

Explant: Mature Cotyledons of RegenS

Plasmid: pPHI256 (GUS, AMVcp, BAR)

Bombardment: 8 cotyledons per plate (8 plates) bombarded twice with 1.8 μm tungsten particles

Culture: Seed germinated 2 days and embryonic axis removed from cotyledon
Cotyledon plated to filters soaked with 0.25 M sorbitol and adaxial surface bombarded twice Cultured on modified B5 medium 2 days
3 day post-bombardment cotyledons cultured on a modified B5 medium containing 2.5 mg/l Basta® for

9 wks
4 wks callusing/embryogenesis (B5 base, 1mg/l
2,4-0 and 0.2 mg/l kinetin)
2 wks embryogeny/embryo development (B5 base, 0,1 mg l NAA)
3 wks embryo maturation (Boi2Y base, no hormones

Rooted in 5 mg/l Basta®
Shoot tips cultures initiated

Results: 60 embryos recovered
11 browned and died during selection
10 abnormal sacrificed for GUS histochemical staining (all negative)
31 abnormal recultured for callus (also GUS negative)
8 normal--5 survived higher selection

[0031] In this experiment, five plants were recovered from culture of bombarded mature cotyledons on modified B5 media containing 2.5 mg/l Basta®. Each plant was identified to contain the AMVcp and BAR genes by the method of polymerase chain reaction amplification, as shown in Table 1. β-glucuronidase enzyme activity was also identified in the five plants by a GUS assay described by Rao, G. and Flynn, P., BioTechniques, Vol. 8, No. 1, pp. 38-40 (1990).

TABLE 1

Alfalfa Plants Recovered on Basta® Selection						
Plant	PCR		GUS[a]			
	AMVcp[b]	BAR[c]	Shoot		Root	
			Assay 1	Assay 2	Assay 1	Assay 2
E1	+	+	3	-	2	2
E2	+	+	-	-	2	1
E3	+	+	-	1	-	N A
E4	+	+	2	-	-	N A
E5	+	+	-	-	-	-

[a]Fluorometric GUS assay expressed as pg /μg total protein.

[b]Oligonucleotides target internal to AMVcp coding region.

[c]Oligonucleotides target CaMV promoter and 5' region of BAR coding region.

[0032] Below, PCR analysis of the parent and progeny is set forth showing 50% were positive for BAR. The first three plants are progeny followed by a maternal plant showing BAR expression, a paternal negative control, maternal plant positive for BAR and controls.

TABLE 2

PCR Analysis of Parent and Progeny Plants			
Sample	Source	BAR	AMV
BOO1E2 x YAE92	Progeny	+	-
BOO1E2 x YAE92	Progeny	-	-
BOO1E3 x YAE92	Progeny	-	-
Maternal BOO1E2	Maternal	+	-
YAE92 Paternal	Paternal	-	-
Maternal BOO1E3	Maternal	+	-
RA3 11-5 + control[a]	NPTII+ AMV+	-	+
RA3 C308 - control		-	-

[a] A description of this positive control is found at Hill, et al., Bio/Technology, 9:373-377 (1991).

[0033] Southern analysis was performed on the parent plants which were found to be clones and were positive by PCR for BAR and AMVcp genes. Thus, it can be seen heritable transformation of plants was achieved.

[0034] In summary, it can be seen that transformation of mature cotyledons from alfalfa can be accomplished through he use of microprojectile bombardment. However, as noted, regeneration is typically poor. Regeneration is dramatically improved by the use of immature cotyledons in transformation and regeneration.

Immature Cotyledons

[0035] Somatic embryogenesis can be direct, where embryos are formed directly from the cells, or indirect where a callus is formed which goes through dedifferentiation.

[0036] Where in the past research has centered on using a particular germplasm source, selecting for genotypes with improved regeneration, recurrent selection to create varieties having improved regeneration, or selection for genes in plant breeding techniques in developing improved regeneration lines, this invention uses an entirely different approach. See, e.g., Mitten, et al., , Crop Science, 24:943 (1984); Seitz, Kris & Bingham, In Vitro, 24:1047 (1988); Brown and Atanassov, Plant Cell Tissue Organ Culture, 4:111-122 (1985). Thus, the invention relates to the use of immature cotyledons to improve regeneration, and thereby transformation of alfalfa.

[0037] The use of immature cotyledons has been found to be an important factor in regeneration. As a seed develops, from about 0-5 days past pollination the seed embryo is globular in shape and generally without form, translucent in color. At about 5 days it demonstrates a heart shaped appearance. The embryo then undergoes rotation, and at about 10 days has a visible cotyledon. The color is translucent to light green, and a scalpel placed behind the cotyledon can almost be visualized. At about 15 days the differentiation of the seed parts has become more distinct, and by 20 days it has a dark green appearance. Beyond 25 days, the dark green color gives away to a yellowing. At 30 days it is creamy white in color. It is at this point that the dormancy process is underway.

[0038] It has been found by the applicant that immature cotyledons providing improved regeneration include those which are formed up to 25 days past pollination. At 5-7 days post-pollination the heart stage is apparent, however, as a practical matter it is difficult to excise the cotyledon portion at this stage and to differentiate it from the other parts of the embryo. The cotyledon can be harvested more easily beginning at about 10 days when it has a translucent to very light green color. The time period between 10-15 days is preferred and provides for considerably improved regeneration results. The most preferable time to excise the cotyledon is at about 10 days past pollination and/or the cotyledon has a translucent to light green color. The light green color can be compared to that found at Panton Color Chart Number PMS372.

[0039] As a result of using immature cotyledons as provided herein, it is possible to regenerate varieties which have never been capable of transformation and regeneration before. Thus, while highly regenerable plants in the past have not always carried the preferred phenotypes, now one may regenerate even elite lines of alfalfa. These elite lines typically have desirable production qualities but notoriously poor regeneration.

[0040] As a further result, when immature cotyledons are used, one can obtain transformation of such elite lines which could not be regenerated previously after introduction of DNA. The transformation may occur by bombardment or the previously known use of agrobacteria, with regeneration now possible.

EXPERIMENT 2

[0041] The typical protocol includes placing the immature cotyledon explant on a modified B_5 medium. After 21-28

days somatic embryos are transferred to MS medium and allowed to mature. Obviously there are a number of variations on this protocol known to those skilled in the art and this is given by way of example. The following shows improved regeneration which correlates to explant age.

[0042] Plants from two varieties were divided into three groups. Six plants from YAE92 were placed into a first group, five plants from YAE92 were placed into a second group, and five plants from YAM93 were placed into a third group. Table 3 below shows the background of each variety. Each group was crossed exclusively within itself. From the resulting plants each raceme is individually identified and its integrity maintained. Harvesting occurs at timed intervals from 0-30 days past pollination, with an early harvest from a numbered raceme and later harvest from the same raceme. By maintaining the integrity of the group and harvesting from a numbered raceme over the time course of the experiment, it can be demonstrated that variation of genotype even within a particular variety does not affect regeneration as long as regeneration is from immature cotyledon. Each of the cotyledons excised at the time course harvest was regenerated. A graph at Figure 3 of the results plots the age of the cotyledon post-pollination on the x-axis and the regeneration response on the y-axis. The results show that even from the same raceme there is increasing regeneration beginning at just after pollination, up to about 15 days past pollination, with declining regeneration up to maturity.

[0043] The scoring and evaluation of the time course harvest is shown in Table 4. Thus, it is clear that age of the cotyledon excised is the critical factor effecting regeneration.

TABLE 3

	Percent Contribution of Germplasm									
	varia	ladak	turk	falc	chil	peru	indian	african	flemish	unk.
YAE92	27	8	4	6	8	--	--	--	47	--
YAM93	23	8	10	8	7	2	--	--	42	--

TABLE 4

Regeneration Shown as Percent Response, From Immature Cotyledons of Different Ages From Controlled Matings Within Three Groups of Alfalfa Plants		
AGE (Days Post-Pollination)	Number Cotyledons Evaluated	Percent Response
6	44	48.
7	38	53.
8	42	52.
9	39	64.
10	52	60.
11	44	61.
12	51	57.
13	53	62.
14	38	55.
15	42	43.
16	38	34.
17	42	27.
18	49	22.
19	59	17.
20	56	14.
21	30	7.
22	45	9.
23	41	7.
24	19	5.
25	68	1.
26	73	1.
27	18	0.
28	9	0.
29	17	0.

TABLE 4 (continued)

Regeneration Shown as Percent Response, From Immature Cotyledons of Different Ages From Controlled Matings Within Three Groups of Alfalfa Plants		
AGE (Days Post-Pollination)	Number Cotyledons Evaluated	Percent Response
30	17	0.
31	15	0.
32	11	0.
33	15	0.
34	9	0.
35	10	0.
36	17	0.
37	18	0.
38	14	0.
39	11	0.
40	12	0.

[0044] Thus, it can be seen that when immature cotyledons are used in regeneration of alfalfa, dramatically improved results occur.

EXPERIMENT 3

[0045] This experiment confirms that it is the immature cotyledon use which provides for the improved regeneration and may be applied to any germplasm source. A number of varieties, including those that have poor or little regeneration were regenerated using immature cotyledons. A minimum of twelve plants of each of the varieties listed in Table 5 were planted and pollinated, with the exception that 15 plants of Grimm (Pi 452472), 30 plants of Mesa Sirsa and 1 plant of RA3 clone were planted and pollinated. Each raceme identified was harvested at about 10-15 days past pollination and at maturity (about 30 days). Immature and mature cotyledons were regenerated as described in Experiment 2.

[0046] The data in Table 5 below demonstrates that use of immature cotyledons substantially improves regeneration even in those varieties which traditionally have poor or no regeneration. Figure 4 graphically displays the differences in regeneration occurring in varieties that are extremely difficult to regenerate. Selected varieties and, in particular, those with the worst regeneration, are shown in terms of percent regeneration of mature cotyledons in the solid bar; and percent regeneration of immature cotyledons, represented by the hashed bar. Use of immature cotyledons resulted in improved regeneration in each instance, including those varieties with no regeneration using mature cotyledons.

TABLE 5

Comparison of Percent of Regeneration of 30 Days Past Pollination Mature Cotyledons With Percent Regeneration of 10-15 Day Post-Pollination Immature Cotyledons				
ALFALFA DESIGNATION	# MATURE COTYLEDONS SAMPLED	% MATURE COTYLEDONS REGENERATING	# IMMATURE COTYLEDONS SAMPLED	% IMMATURE COTYLEDONS REGENERATING
Grimm (Pi 452472)	206	0	223	15
Norseman	152	28	198	37
Lahontan	157	2	184	30
Turkistan (Pi 86696)	176	8	186	18
Teton	145	3	175	16
Pi251689	140	0	129	21
Caliverde 65	138	0	167	27
Buffalo	127	1	158	20
Cody	161	0	183	31
Hairy Peruvian	147	4	166	18
Hairy Peruvian (B16-PLH)	150	0	173	22

TABLE 5 (continued)

Comparison of Percent of Regeneration of 30 Days Past Pollination Mature Cotyledons With Percent Regeneration of 10-15 Day Post-Pollination Immature Cotyledons				
ALFALFA DESIGNATION	# MATURE COTYLEDONS SAMPLED	% MATURE COTYLEDONS REGENERATING	# IMMATURE COTYLEDONS SAMPLED	% IMMATURE COTYLEDONS REGENERATING
Mesa Sirsa	243	0	262	17
Sonora	110	11	127	25
DuPuits	138	6	145	24
Iroquois	143	0	158	26
Vernal	152	22	161	34
Culver	170	0	173	23
Agate	135	0	155	19
Ramsey	121	0	181	24
El Unico	149	0	190	28
RegenS/ RA3	43	54	63	72
YAM93	164	0	196	34
YAE92	179	0	187	27

EXPERIMENT 4

[0047] Three separate tests were conducted to determine if immature embryos could be transformed.

[0048] In the first test, cotyledons were bombarded with pPHI413 (see Figure 5), as above, and levels of GUS expression assayed. Forty-two samples were bombarded. Optimum expression occurred 48 to 72 hours post bombardment where 26 of the 42 samples expressed GUS with a mean of 1.7 pg/μg total protein. Five days post bombardment 6 of 30 samples showed an average of 2 pg/μg total protein, while at 17 days post bombardment 3 of 30 samples showed an average of 2 pg/μg total protein.

[0049] In the second, the effect of bombardment on alfalfa regeneration under selection was studied. Immature cotyledons of Regen S were harvested 11 days post-pollination. Cotyledons were excised from the embryo, bombarded three times with the plasmid pPHI251 (Figure 1), adsorbed to tungsten particles, and cultured on modified B5 media containing 25 mg/l kanamycin sulfate. Somatic embryos were harvested approximately two months after treatment, allowed to desiccate on M5 media for two months, and germinated on MS media containing 100 mg/l kanamycin sulfate. Leaf tissue was harvested and assayed for neomycin phosphotransferase (NPTII) activity. The results are shown in Table 6.

Table 6

Plant	NPTII activity pg/μg total protein	AMVcp (elisa)
CBX106	3	+
CBX107	2	-
CBY107	5	-
CBY108	4	-
CBZ108	1	-
CBX112	1	+
CBY112	3	-
CBZ112	1	-
CBA112	1	-
CBX115	2	-
CBX116	2	+
CBY116	3	-
CBX117	3	-
1 Regen S 3-11	13	-
2 Regen S 3-11	10	-

Table 6 (continued)

Plant	NPTII activity pg/µg total protein	AMVcp (elisa)
3 Regen S 3-11	9	-
Regen S Negative Control[a]	0	-
Rambler Positive Control[b]	4	+

[a] The negative control was bombarded with TE buffer-treated tungsten particles and regenerated on media not containing kanamycin.

[b] Rambler Positive Control was a previously identified transgenic alfalfa plant shown to contain and express the neomycin phosphotransferase gene (Hill et al., Bio/Technology, 9:373-377 (1991)).

[0050] In the third test, yet another embodiment of the invention is demonstrated and the affect of bombardment on the regeneration of transformed elite alfalfa varieties was examined. Immature cotyledons were excised from 11 day post-pollination embryos. Somatic embryos were regenerated. Somatic embryos were bombarded five times with tungsten particles adsorbed with the plasmid pPHI251 (Figure 1) and cultured on modified B5 media containing 25 mg/l kanamycin sulfate. Embryos were subcultured at 20 days post-bombardment to fresh modified B5 media containing 25 mg/l kanamycin sulfate. Green somatic embryos were harvested 50 days post bombardment and matured on MS medium containing 100 mg/l kanamycin sulfate. Leaf samples were taken at 80 days post-bombardment and assayed for neomycin phosphotransferase activity. The results are shown in Table 7.

Table 7

Yam93 Regenerant	NPTII Activity (pg/µg Total Protein)
CB93.1	11
CB93.2	13
CB93.3	3
CB93.4	8
CB93.5	4
CB93.6	9
Yam93 negative control[a]	0
Rambler 10-1-1[b]	2

[a] The negative control plant was regenerated from bombarded immature cotyledons bombarded with TE-buffer treated tungsten particles.

[b] Rambler 10-1-1 was a previously identified transgenic plant shown to contain and express the neomycin phosphotransferase gene. [Hill, et al., Bio/technology, 9:373-377 (1991)].

[0051] The latter test demonstrates that when immature cotyledons are used to form somatic embryos, and then those embryos are bombarded, even more plants are recovered. Furthermore, the resulting plant has been found to retain this ability to regenerate. Elite varieties can not only be regenerated, but also retain this property.

[0052] It can further be seen that bombardment of the immature embryos or somatic embryos does not adversely affect regeneration and that DNA is expressed in these now regenerable cells and plants.

[0053] The foregoing demonstrates transformation of Medicago sativa, transformation with particle acceleration, and that substantially improved regeneration of Medicago sativa is possible by the use of immature cotyledons.

[0054] Regeneration of varieties not previously regenerated or with very poor regeneration is achieved. Thus, transformation of these same varieties is now possible.

[0055] Thus, it can be seen the invention accomplishes its objectives.

Claims

1. A process for regeneration of alfalfa comprising initiating somatic embryogenesis of immature cotyledon cells of alfalfa up to 25 days past pollination.

2. A process as claimed in claim 1 wherein the immature cotyledon cells are 10 to 15 days past pollination.

3. A process as claimed to claim 1 wherein the immature cotyledon cells are about 10 days past pollination.

4. A process as claimed in any one of the preceding claims wherein the cotyledon is translucent to light green in colour.

5. A process as claimed in any one of the preceding claims wherein an elite variety of alfalfa is regenerated.

6. A process as claimed in claim 5 wherein the variety is selected from the varieties listed in Table 5.

7. A process as claimed in any one of the preceding claims wherein an immature cotyledon is excised from seed embryos of alfalfa, the cotyledon placed in contact with an auxin to induce cell division and growth, causing somatic embryogenesis from the immature cotyledon.

8. A process as claimed in any one of the preceding claims followed by culturing the resulting somatic embryo into a mature alfalfa plant.

9. A process of obtaining expression of foreign DNA in cells of alfalfa comprising: attaching the foreign DNA to carrier particles; physically accelerating the particles at the cells to bombard the cells with the particles having the foreign DNA such that at least some of the foreign DNA is inserted into the interior of at least some of the cells and confirming expression of the foreign DNA in the cells, wherein the alfalfa cells are cells of an immature cotyledon up to 25 days past pollination or cells of a somatic embryo derived therefrom.

10. A process as claimed in claim 9 wherein the cells are bombarded one or two times.

11. A process as claimed in claim 9 or claim 10 wherein the cells employed are cells of immature cotyledons 10 to 15 days past pollination.

12. A process as claimed in claim 9 or claim 10 wherein the cells employed are cells of immature cotyledons about 10 days past pollination.

13. A process as claimed in any one of claims 9 to 12 wherein the cells employed are cells of immature cotyledons translucent to light green in colour.

14. A process as claimed in any one of claim 9 to 13 followed by cultivating the cells with foreign DNA into alfalfa plants.

15. A process as claimed in claim 14 wherein the foreign DNA is attached to carrier particles, the particles are accelerated at an immature cotyledon such that at least some of the DNA is introduced into the interior of the immature cotyledon cells, the tissue is cultured on a growth promoting medium and the resulting growth cultivated into a whole mature alfalfa plant containing the introduced DNA.

16. A process as claimed in any one of claims 9 to 15 wherein the DNA is transformed into cells of an elite variety of alfalfa.

17. A process of transforming foreign DNA into alfalfa plants comprising initiating somatic embryogenesis of immature cotyledon cells of alfalfa up to 25 days past pollination, introducing the DNA into cells of the embryos by microprojectile bombardment or by use of Agrobacterium tumefaciens and cultivating the cells with the DNA into alfalfa plants.

18. A process as claimed in claim 17 wherein the immature cotyledon cells are 10 to 15 days past pollination.

19. A process as claimed in claim 17 wherein the immature cotyledon cells are about 10 days past pollination.

20. A process as claimed in any one of claims 17 to 19 wherein the cells employed for initiation of somatic embryogenesis are cells of immature cotyledons translucent to light green in colour.

21. A process as claimed in any one of claims 17 to 20 wherein an elite variety of alfalfa is transformed.

Patentansprüche

1. Verfahren zur Regeneration von Luzerne, umfassend das Initiieren der somatischen Embryogenese unreifer Keimblattzellen der Luzerne bis zu 25 Tagen nach der Bestäubung.

2. Verfahren nach Anspruch 1, dadurch **gekennzeichnet,** daß sich die unreifen Keimblattzellen im Zustand von 10 bis 15 Tagen nach der Bestäubung befinden.

3. Verfahren nach Anspruch 1, dadurch **gekennzeichnet,** daß sich die unreifen Keimblattzellen im Zustand von etwa 10 Tagen nach der Bestäubung befinden.

4. Verfahren nach einem der vorhergehenden Ansprüche, dadurch **gekennzeichnet,** daß das Keimblatt für Licht von grüner Farbe transluzent ist.

5. Verfahren nach einem der vorhergehenden Ansprüche, dadurch **gekennzeichnet**, daß eine Luzerne-Elitesorte regeneriert wird.

6. Verfahren nach Anspruch 5, dadurch **gekennzeichnet,** daß die Sorte aus den in Tabelle 5 aufgelisteten Sorten ausgewählt ist.

7. Verfahren nach einem der vorhergehenden Ansprüche, dadurch **gekennzeichnet,** daß ein unreifes Keimblatt aus Luzernesamenembryos herausgeschnitten wird, daß das Keimblatt in Kontakt mit einem Auxin gebracht wird, um Zellteilung und Wachstum zu induzieren, wodurch die somatische Embryogenese ausgehend von dem unreifen Keimblatt bewirkt wird.

8. Verfahren nach einem der vorhergehenden Ansprüche, gefolgt von der Kultivierung des entstehenden somatischen Embryos zu einer reifen Luzernepflanze.

9. Verfahren zum Erhalten der Expression fremder DNA in Luzernezellen, umfassend die folgenden Stufen: Bindung der Fremd-DNA an Trägerteilchen; physikalische Beschleunigung der Teilchen in Richtung der Zellen, um die Zellen mit den Teilchen, die die fremde DNA besitzen, zu bombardieren, so daß wenigstens ein Teil der Fremd-DNA in das Innere wenigstens einiger der Zellen gebracht wird und Bestätigung der Expression der fremden DNA in den Zellen, wobei die Luzernezellen Zellen eines unreifen Keimblatts bis zu 25 Tagen nach der Bestäubung oder Zellen eines davon abgeleiteten somatischen Embryos sind.

10. Verfahren nach Anspruch 9, dadurch **gekennzeichnet,** daß die Zellen ein- oder zweimal bombardiert werden.

11. Verfahren nach Anspruch 9 oder 10, dadurch **gekennzeichnet,** daß die verwendeten Zellen Zellen unreifer Keimblätter sind, die sich im Zustand von 10 bis 15 Tagen nach der Bestäubung befinden.

12. Verfahren nach Anspruch 9 oder 10, dadurch **gekennzeichnet,** daß die verwendeten Zellen Zellen unreifer Keimblätter sind, die sich im Zustand von etwa 10 Tagen nach der Bestäubung befinden.

13. Verfahren nach einem der Ansprüche 9 bis 12, dadurch **gekennzeichnet,** daß die verwendeten Zellen Zellen unreifer Keimblätter sind, die für Licht grüner Farbe transluzent sind.

14. Verfahren nach einem der Ansprüche 9 bis 13, gefolgt von der Kultivierung der Fremd-DNA-enthaltenden Zellen in Luzernepflanzen.

15. Verfahren nach Anspruch 14, dadurch **gekennzeichnet,** daß die Fremd-DNA an Trägerpartikel gebunden ist, daß die Teilchen in Richtung eines unreifen Keimblatts beschleunigt werden, so daß wenigstens ein Teil der DNA in das Innere der unreifen Keimblattzellen eingeführt wird, daß das Gewebe auf einem wachstumsfördernden Medium kultiviert wird und daß das resultierende Wachstum zu einer ganzen reifen Luzernepflanze führt, die die eingeführte DNA enthält.

16. Verfahren nach einem der Ansprüche 9 bis 15, dadurch **gekennzeichnet,** daß die DNA in Zellen einer Luzerne-Elitesorte transformiert wird.

17. Verfahren zur Transformation von Fremd-DNA in Luzerneplanzen, umfassend das Initiieren der somatischen Embryogenese unreifer Keimblattzellen der Luzerne bis zu 25 Tagen nach der Bestäubung, das Einführen der DNA in Zellen der Embryos durch Mikroprojektilbombardierung oder durch Verwendung von Agrobacterium tumefaciens und Kultivieren der DNA-enthaltenden Zellen zu Luzernepflanzen.

18. Verfahren nach Anspruch 17, dadurch **gekennzeichnet,** daß sich die unreifen Keimblattzellen im Zustand von 10 bis 15 Tagen nach der Bestäubung befinden.

19. Verfahren nach Anspruch 17, dadurch **gekennzeichnet,** daß sich die unreifen Keimblattzellen im Zustand von etwa 10 Tagen nach der Bestäubung befinden.

20. Verfahren nach einem der Ansprüche 17 bis 19, dadurch **gekennzeichnet,** daß die zur Initiierung der somatischen Embryogenese verwendeten Zellen Zellen unreifer Keimblätter sind, die für Licht grüner Farbe transluzent sind.

21. Verfahren nach einem der Ansprüche 17 bis 20, dadurch **gekennzeichnet,** daß eine Luzerne-Elitesorte transformiert wird.

Revendications

1. Procédé pour la régénération de luzerne comprenant l'induction de l'embryogenèse somatique de cellules de cotylédons immatures de luzerne jusqu'à 25 jours après pollinisation.

2. Procédé tel que revendiqué dans la revendication 1, dans lequel les cellules de cotylédons immatures ont 10 à 15 jours après pollinisation.

3. Procédé tel que revendiqué dans la revendication 1, dans lequel les cellules de cotylédons immatures ont environ 10 jours après pollinisation.

4. Procédé tel que revendiqué dans l'une quelconque des revendications précédentes, dans lequel le cotylédon est translucide à la lumière de couleur verte.

5. Procédé tel que revendiqué dans l'une quelconque des revendications précédentes, dans lequel une variété d'élite de luzerne est régénérée.

6. Procédé tel que revendiqué dans la revendication 5, dans lequel la variété est choisie parmi les variétés de la liste du tableau 5.

7. Procédé tel que revendiqué dans l'une quelconque des revendications précédentes, dans lequel un cotylédon immature est excisé à partir d'embryons de graine de luzerne, le cotylédon est placé en contact avec une auxine pour induire la division et la croissance cellulaire, provoquant l'embryogenèse somatique à partir du cotylédon immature.

8. Procédé tel que revendiqué dans l'une quelconque des revendications précédentes, suivi de la culture de l'embryon somatique obtenu en un plant de luzerne mature.

9. Procédé pour obtenir l'expression d'ADN étranger dans des cellules de luzerne comprenant les étapes consistant à : fixer de l'ADN étranger à des particules support ; accélérer physiquement les particules au niveau des cellules pour bombarder les cellules avec les particules ayant l'ADN étranger, de telle sorte qu'au moins une partie de l'ADN étranger s'insère à l'intérieur d'au moins certaines cellules, et confirmer l'expression de l'ADN étranger dans les cellules, dans lequel les cellules de luzerne sont des cellules d'un cotylédon immature ayant jusqu'à 25 jours après pollinisation ou des cellules d'un embryon somatique dérivé de celles-ci.

10. Procédé tel que revendiqué dans la revendication 9, dans lequel les cellules sont bombardées une ou deux fois.

11. Procédé tel que revendiqué dans la revendication 9 ou 10, dans lequel les cellules employées sont des cellules de cotylédons immatures ayant 10 à 15 jours après pollinisation.

12. Procédé tel que revendiqué dans la revendication 9 ou 10, dans lequel les cellules employées sont des cellules de cotylédons immatures ayant environ 10 jours après pollinisation.

13. Procédé tel que revendiqué dans l'une quelconque des revendications 9 à 12, dans lequel les cellules employées sont des cellules de cotylédons immatures translucides à la lumière de couleur verte.

14. Procédé tel que revendiqué dans l'une quelconque des revendications 9 à 13, suivi de la culture des cellules avec de l'ADN étranger en plants de luzerne.

15. Procédé tel que revendiqué dans la revendication 14, dans lequel l'ADN est fixé à des particules support, les particules sont accélérées au niveau d'un cotylédon immature de telle sorte qu'au moins une partie de l'ADN s'introduise à l'intérieur des cellules de cotylédons immatures, le tissu est cultivé sur un milieu favorisant la croissance et le produit de cette croissance est cultivé en un plant de luzerne entier mature contenant l'ADN introduit.

16. Procédé tel que revendiqué dans l'une quelconque des revendications 9 à 15, dans lequel l'ADN est transformé dans des cellules d'une variété d'élite de luzerne.

17. Procédé de transformation d'ADN étranger dans des plants de luzerne comprenant les étapes consistant à induire l'embryogenèse somatique de cellules de cotylédons immatures de luzerne ayant jusqu'à 25 jours après pollinisation, introduire l'ADN dans des cellules des embryons par bombardement de microprojectiles ou par utilisation d'Agrobacterium tumefaciens et cultiver des cellules avec l'ADN en plants de luzerne.

18. Procédé tel que revendiqué dans la revendication 17, dans lequel les cellules de cotylédons immatures ont environ 10 à 15 jours après pollinisation.

19. Procédé tel que revendiqué dans la revendication 17, dans lequel les cellules de cotylédons immatures ont environ 10 jours après pollinisation.

20. Procédé tel que revendiqué dans l'une quelconque des revendications 17 à 19, dans lequel les cellules employées pour l'induction de l'embryogenèse somatique sont des cellules de cotylédons immatures translucides à la lumière de couleur verte.

21. Procédé tel que revendiqué dans l'une quelconque des revendications 17 à 20, dans lequel on transforme une variété d'élite de luzerne.

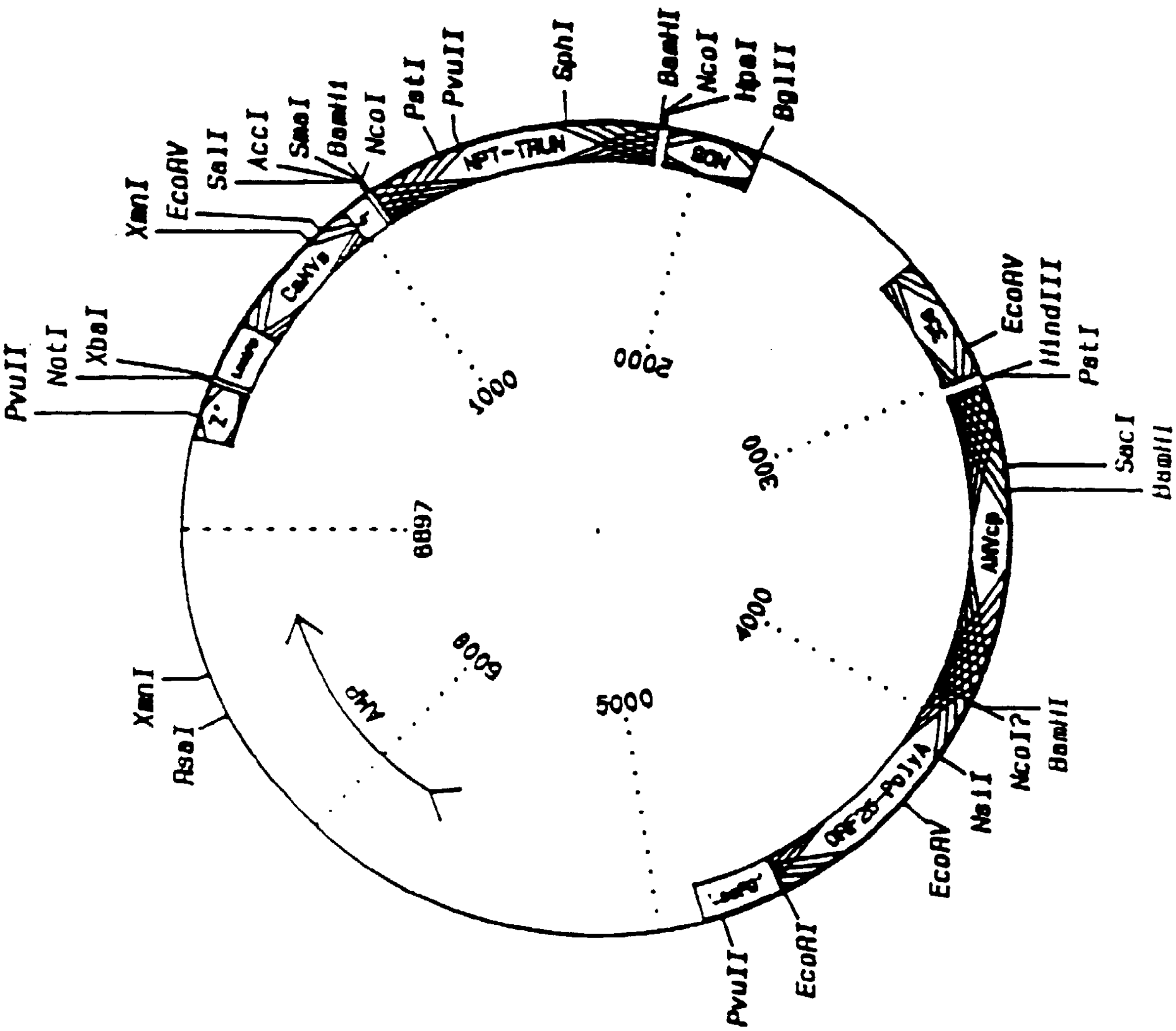
PvuII
NotI
XbaI
XmnI
EcoRV
SalI
AccI
SmaI
BamHI
NcoI
PstI
PvuII
SphI
BamHI
NcoI
HpaI
BglII
EcoRV
HindIII
PstI
SacI
BamHI
NcoI?
BamHI
NsiI
EcoRV
EcoRI
PvuII
XmnI
AsaI
NPT-TRUN
CaMVs
AMVcp
ORF25-PolyA
AMP
1000
2000
3000
4000
5000
6000
6897

Figure 1

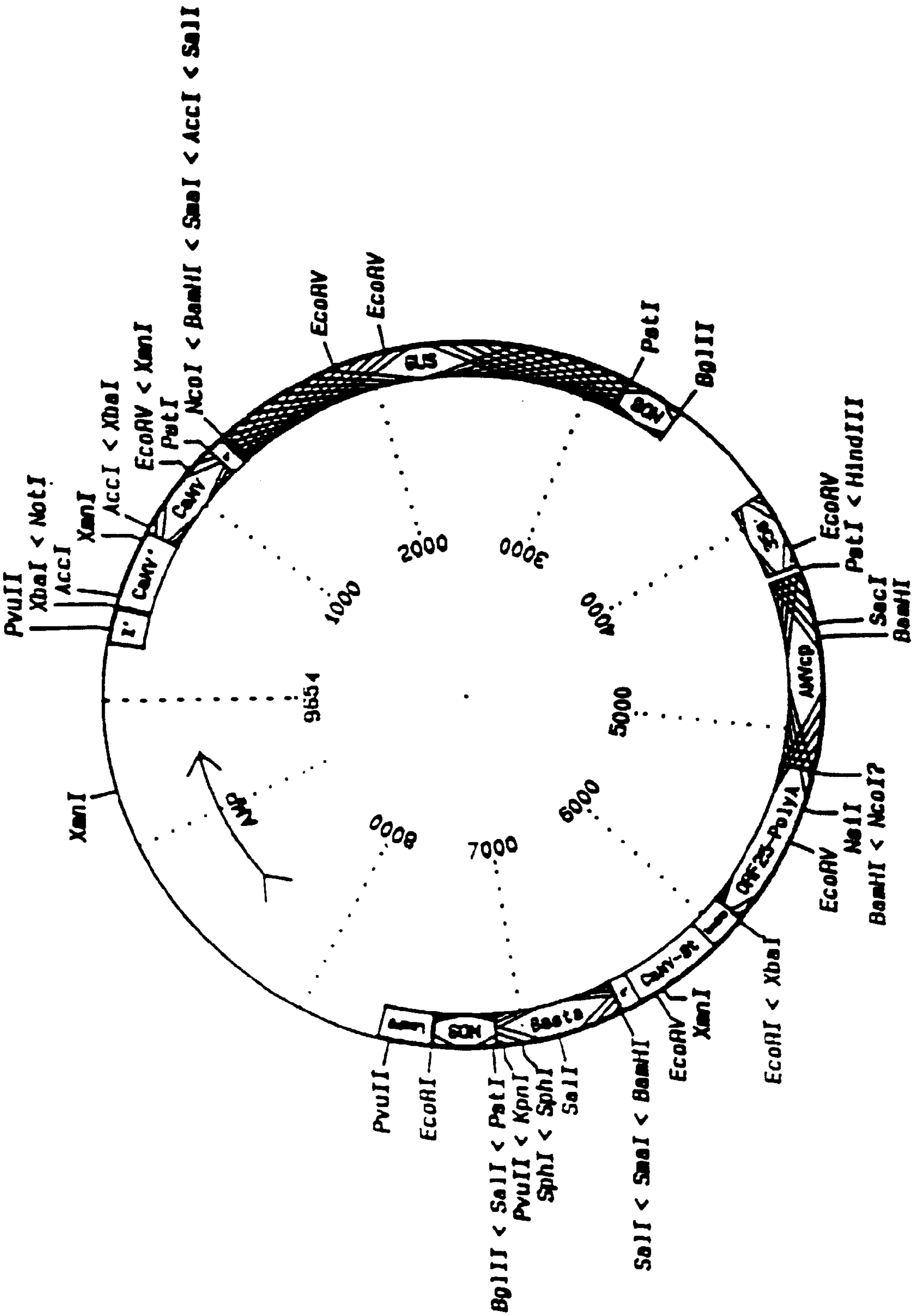
PvuII
XbaI < NotI
AccI
XmnI
AccI < XbaI
EcoRV < XmnI
PstI
NcoI < BamHI < SmaI < AccI < SalI
EcoRV
EcoRV
PstI
BglII
EcoRV
PstI < HindIII
SacI
BamHI
NsII
BamHI < NcoI?
EcoRV
EcoRI < XbaI
XmnI
EcoRV
SalI < SmaI < BamHI
SalI
SphI < SphI
PvuII < KpnI
BglII < SalI < PstI
EcoRI
PvuII
XmnI
1000
2000
3000
4000
5000
6000
7000
8000
9654

Figure 2

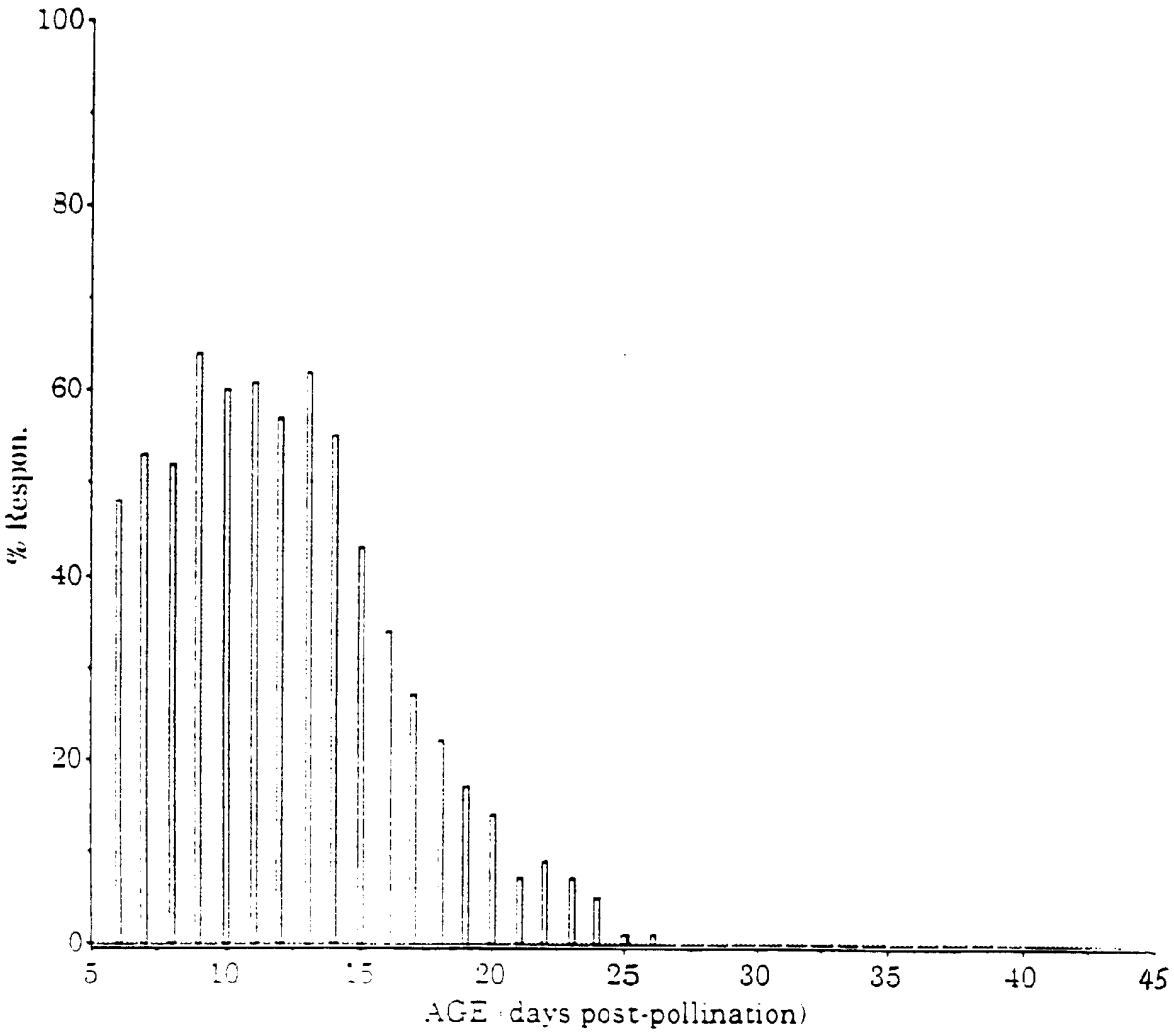
100
80
60
40
20
0
% Respon.
5
10
15
20
25
30
35
40
45
AGE (days post-pollination)

Figure 3

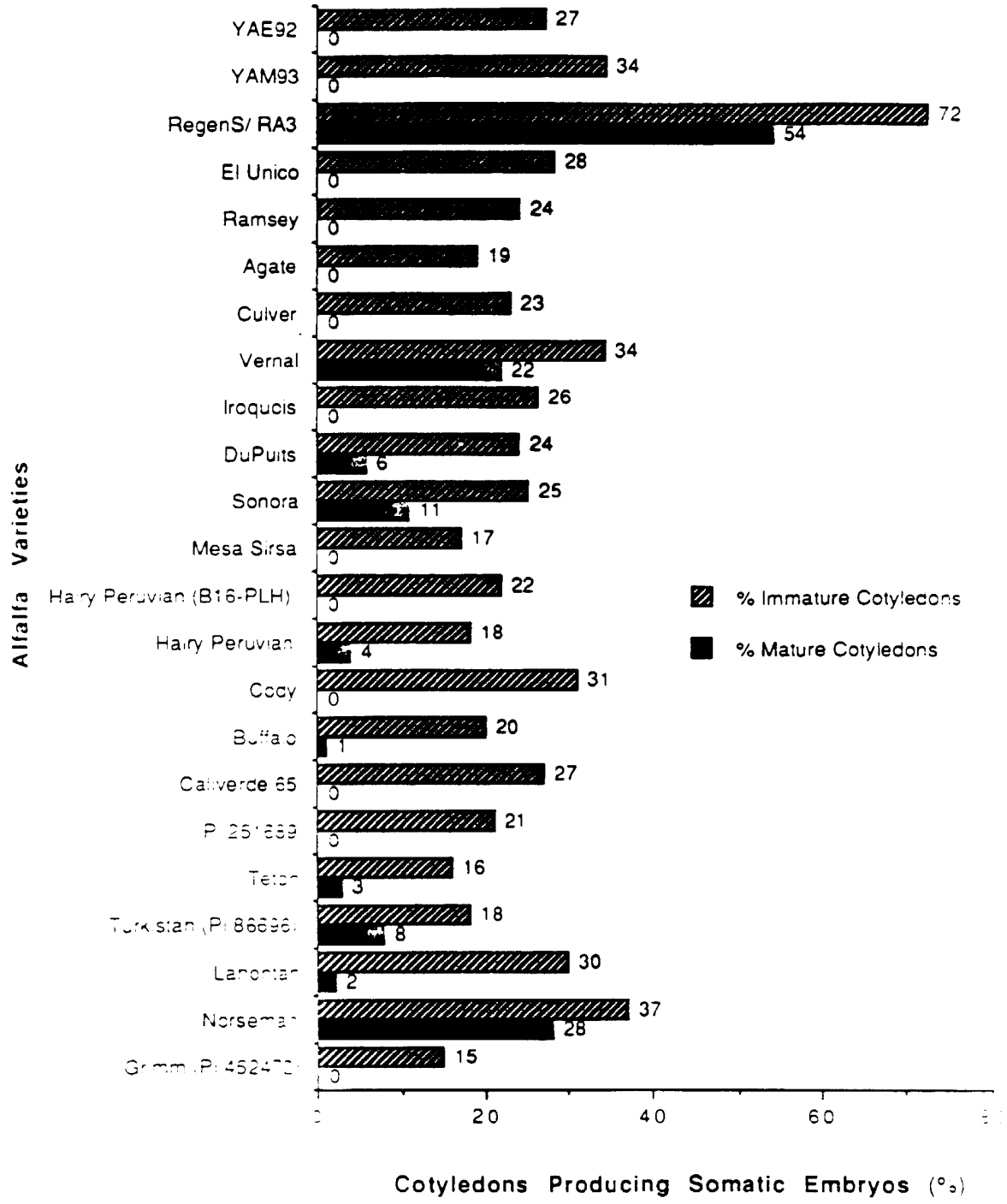
Alfalfa Varieties
YAE92
YAM93
RegenS/ RA3
El Unico
Ramsey
Agate
Culver
Vernal
Iroquois
DuPuits
Sonora
Mesa Sirsa
Hairy Peruvian (B16-PLH)
Hairy Peruvian
Cody
Buffalo
Caliverde 65
P 251639
Teton
Turkistan (PI 86696)
Lahontan
Norseman
Grimm (PI 452472)
27
0
34
0
72
54
28
0
24
0
19
0
23
0
34
22
26
0
24
6
25
11
17
0
22
0
18
4
31
0
20
1
27
0
21
0
16
3
18
8
30
2
37
28
15
0
% Immature Cotyledons
% Mature Cotyledons
0
20
40
60
Cotyledons Producing Somatic Embryos (%)

Figure 4

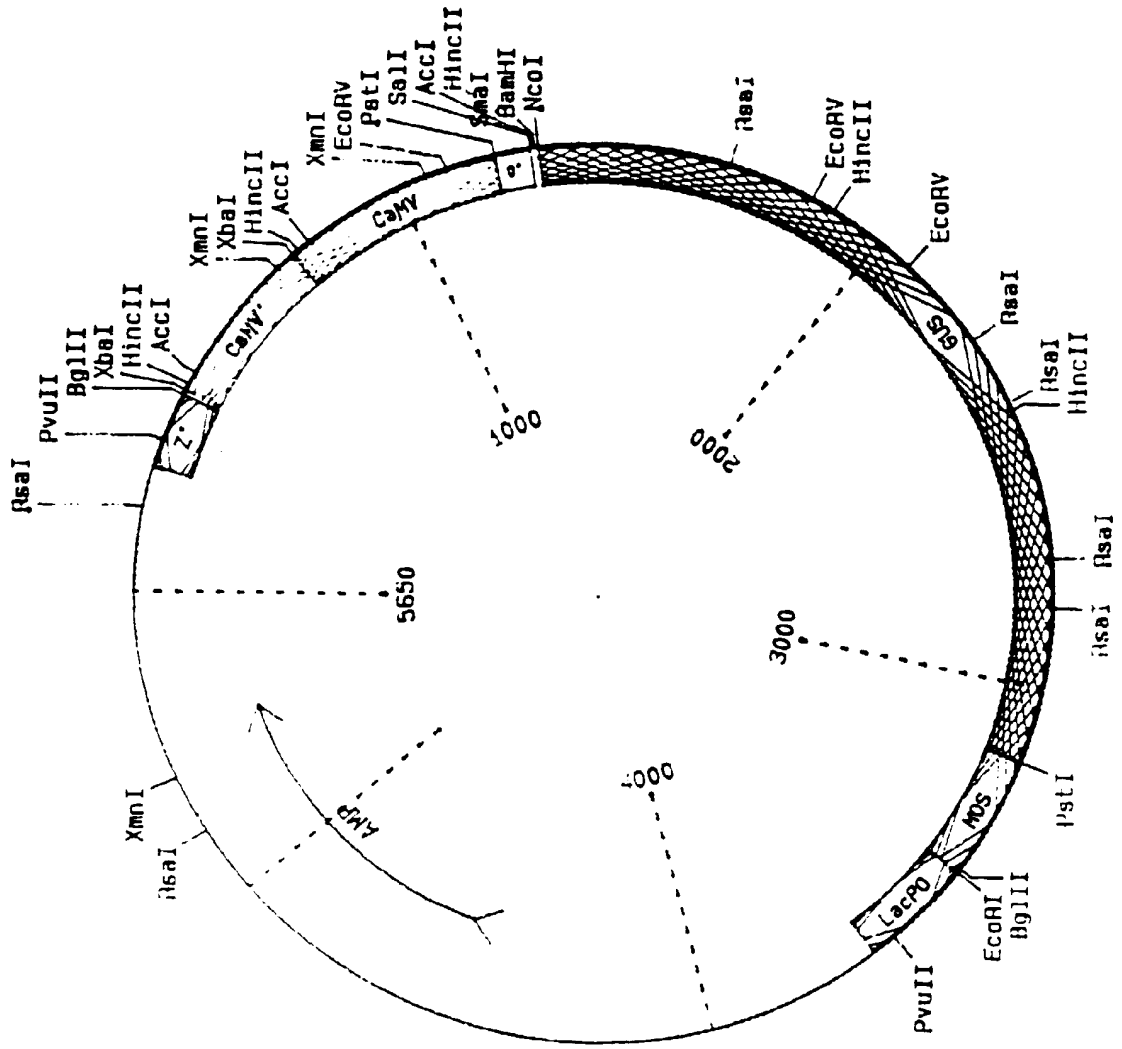
RsaI
PvuII
BglII
XbaI
HincII
AccI
XmnI
XbaI
HincII
AccI
XmnI
EcoRV
PstI
SalI
AccI
HincII
SmaI
BamHI
NcoI
RsaI
EcoRV
HincII
EcoRV
RsaI
RsaI
HincII
RsaI
RsaI
PstI
EcoRI
BglII
PvuII
XmnI
RsaI
CaMV
CaMV
GUS
NOS
LacPO
AMP
5650
1000
2000
3000
1000

Figure 5